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Detection of ryanodine receptor antibodies.

The present invention relates to methods, kits and compositions for the detection of ryanodine receptor antibodies in patient serum samples. The invention also relates to a method for the manufacture of a pharmaceutical agent for the prevention and/or treatment of the disease myasthenia gravis, and a method of myasthenia gravis prognosis.

Myasthenia gravis (MG) is a disease of the neuromuscular junction caused and characterised by antibodies against the Acetyl Choline Receptor (AChR) of the muscle endplate. The AChR antibodies cause a complement mediated damage to the postsynaptic part of the muscle endplate leading to impaired neuromuscular transmission, muscular weakness and fatiguability (Lindstrom, J., D. Schelton, and Y. Fujii. 1988, *Adv. Immunol.* 42: 233-284). However, some MG patients, mainly with thymoma, have antibodies also against other muscle antigens.

Electron microscopic studies have shown that MG sera bind to an unidentified protein in sacroplasmic reticulum (SR) membranes (Mendell, J.R., J.N. Whitaker, and W.K. Engel. 1973, *J. Immunol.* 111: 847-856; Flood, P.R., R. Bjugn, N.E. Gilhus, H. Hofstad, R. Matre, and J.A. Aarli. 1987, *Ann. N.Y. Acad. Sci.* 505: 732-734). Mygland et al., showed that MG sera from thymoma patients stained a high molecular weight protein in a SR preparation (Mygland, Å., O.B. Tysnes, J.A. Aarli, P.R. Flood, and N.E. Gilhus. 1992, *J. Neuroimmunol.* 37: 1-7), which was later identified to be

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identical to the Ca^{2+} release channel of SR, i.e. the ryanodine receptor (RyR) (Mygland, Å., O.B. Tysnes, R. Matre, P. Volpe, J.A. Aarli, and N.E. Gilhus. 1992, *Ann. Neurol.* 32: 589-591).

The RyR is a Ca^{2+}-release channel located in the SR of striated muscle. It plays an essential role in muscle contraction by responding to sarcolemma depolarisation with the opening of the ion channel and the release of Ca^{2+} from SR to contractile proteins in the myoplasm (Coronado, R., J. Morrissette, M. Sukhareva, and D.M. Vaugham. 1994, *Am. J. Physiol.* 266: c1485-c1504).

RyR antibodies were found in approximately half of thymoma MG patients but not in non-thymoma late-onset MG, early onset MG, blood-donors or patients with other autoimmune diseases (Mygland, Å., O.B. Tysnes, R. Matre, P. Volpe, J.A. Aarli, and N.E. Gilhus. 1992, *Ann. Neurol.* 32: 589-591). The RyR antibodies are mainly of the IgG1 and IgG3 subclasses (Mygland, Å., O.B. Tysnes, J.A. Aarli, R. Matre, and N.E. Gilhus. 1993, *J. Autoimmunity.* 6: 507-515), and stain both the skeletal and cardiac form of the RyR (Mygland, Å., O.B. Tysnes, R. Matre, J.A. Aarli, and N.E. Gilhus. 1994, *Autoimmunity* 17: (4) 327-31).

The presence of RyR antibodies correlate to MG severity and even death with a nice correlation also to RyR-antibody levels. Thymoma MG patients with RyR antibodies have in contrast to RyR negative patients often heart disease leading to sudden cardiac arrest. Others die of respiratory failure due to the neuromuscular weakness (Mygland, Å., O.B. Tysnes, R. Matre, J.A. Aarli, and N.E. Gilhus. 1994, *Autoimmunity* 17: (4) 327-31.; Mygland, Å., J.A. Aarli, R. Matre, and N.E. Gilhus. 1994, *J. Neurol. Neurosurg. Psychiatry.* 57: 843-846; Skeie, G.O., E. Bartoccioni, A. Evoli, J.A. Aarli, and N.E. Gilhus. 1996, *Eur. J. Neurol.* 3: 136-140).

The mechanisms leading to muscular fatiguability in MG patients might be more complex than what can be explained

from the AChR antibody model alone. Pagala and others have found evidence for disordered (Pagala, M., N.V. Nandakumar, S.A.T. Venkatachari, K. Ravindran, T. Namba, and D. Grob. 1990, *Muscle and Nerve* 13: 1012-1022; Pagala, M., N.V. Nandakumar, S.A.T. Venkatachari, K. Ravindran, B. Amaladevi, T. Namba, and D. Grob. 1993, *Muscle and Nerve* 16: 911-921) excitation-contraction coupling, for which the RyR is essential, in individual MG patients. The MG patients RyR antibodies are able to inhibit binding of ryanodine to the RyR indicating that the RyR antibodies lock the RyR in the closed position (Skeie, G.O., P.K. Lunde, O.M. Sejersted, Å. Mygland, J.A. Aarli, and N.E. Gilhus. 1998, *Muscle and Nerve* 21: 329-33). Patients with inhibiting antibodies had a more severe disease than patients without such antibodies (Skeie et al., 1998).

MG sera containing striational antibodies have been shown to inhibit caffeine induced Ca^{2+} release in rat muscle cells (Asako et al., 1997). Experimental RyR antibodies can affect RyR function in vitro (Treves S, Chiozzi P, Zorzato F (1993) Biochem J 291, 757-763) and a rat strain which develop spontaneous thymomas and RyR antibodies have muscular weakness and fatiguability resembling MG without detectable AChR antibodies (Iwasa, K., K. Komai, T. Asaka, E. Nitta, and M. Takamori, *Ann. N.Y. Acad. Sci.* 1998:841; 542-545). These studies might indicate a direct pathogenetic role for the RyR antibodies in MG, and thus RyR antibodies do not only have a function as a disease marker.

In this study we have identified the main immunogenic region on the RyR for MG patients antibodies. We show that the antibodies reactive with this part of the RyR are able to inhibit Ca^{2+} release from SR vesicles in vitro, and using a biosensor we were able to study the real time interaction between the RyR antibodies and the RyR fusion protein containing the MIR for the RyR antibodies in MG sera.

The present invention relates to a method for the detection of ryanodine receptor antibodies in patient serum samples, said antibodies being associated with the disease myasthenia gravis, said method comprising the following steps;

(a) obtaining a serum sample from a patient suspected of having myasthenia gravis or being at risk for the development of said disease;

(b) contacting said serum sample with a composition of fusion proteins comprising the following sequences: SEQ ID NO 1 or SEQ ID NO 2;

c) detecting fusion protein - antibody complex formation, wherein said detected complexes indicate the presence of ryanodine receptor antibodies.

Further, the invention relates to the use of the fusion proteins comprising the sequences SEQ ID NO 1 or SEQ ID NO 2 for the detection of RyR antibodies.

Also comprised by the invention is a diagnostic kit for the detection of ryanodine receptor antibodies in patient serum samples, said antibodies being associated with the disease myasthenia gravis, said kit comprising fusion proteins having the following sequences; SEQ ID NO 1 or SEQ ID NO 2.

A preferred embodiment of the invention relates to a diagnostic kit, wherein the immunodetection reagent is a radiolabelled reagent.

The presence of pc2 or pc25 fusion protein antibodies is indicative of the presence of a thymoma.

The present invention also relates to a composition of fusion proteins useful for the detection of ryanodine receptor antibodies, which are associated with the disease myasthenia gravis, said proteins being selected from the group of proteins having a sequence of SEQ ID NO 1 or SEQ ID NO 2, or a combination of said sequences, and to a method for the manufacture of a pharmaceutical agent for the prevention and/or treatment of the disease myasthenia

gravis, wherein said agent are administered to a patient in need thereof, in a amount sufficient to inhibit the binding of ryanodine receptor antibodies to the ryanodine receptor, said composition comprising a panel of fusion proteins having sequences SEQ ID NO 1 and/or SEQ ID NO 2, and to a method of myasthenia gravis prognosis which involves the determination of the presence of RyR antibodies wherein the RyR antibodies are identified by the use of the fusion proteins pc2 and pc25.

The invention will now be further described with respect to the following examples and the accompanying figures, in which:

Figure 1A shows in diagrammatic form the Ryanodine receptor fusion protein strategy. Figure 1B shows the induced *E. coli* extracts loaded in the gel. Proteins were separated by electrophoreses, blotted into nitrocellulose and stained with Ponceau Red. The lower row shows the Western blot staining.

Figure 2A shows a Ryanodine receptor fusion protein strategy designed to narrow the immunopositive region. Figure 2B shows the induced *E. coli* extracts loaded in the gel. The lower row shows the Western blot staining (stained with Ab from patients).

Figure 3 shows the fragments pc2, pc2A and pc2B blotted onto nitrocellulose membranes and stained with Ab from patient

Figure 4 shows the characterisation of the binding between MG patients' antibodies and the pc2 fusion protein. Figure 4A shows the curves from injections with anti-path antibody in concentrations ranging from 2.5-25 mg/ml (Puickinject, flowrate 5 µl/min in HBS, pH 7.4) in the flowcell with bound pc2. The maximal response was 400 RU.

Figure 4B shows the results from injections of IgG fractions from MG patients and controls. Non-covalently bound proteins were removed and the sensor ship regenerated by injections of 5 µl of 0.5 % SDS and/or 3 M guanidinium

chloride in 5 mM Tris, pH 8, between injections of serum or IgG samples.

Figure 4C shows the results from the sandwich assay. The anti-path antibody was immobilised on the sensor ship, and a) the injection of electroeluted pc2 Ry1 fusion protein gave a signal of 60 RU, and b) the second injection of Ry1 antibody positive MG IgG lead to an additional increase of 180 RU which is consistent with a Ab binding stochimetry of 1.

Figur 5 shows 4-chloro-m-cresol (4-cmc) induced Ca^{2+} release from skeletal muscle SR. Ca^{2+} concentrations are represented by the A710-790 of the Ca2+ indicator antipyrolazo III. SR was added to the cuvette followed by 6 consecutive additions of 20 nmol $CaCl_2$ to load the vesicles with Ca^{2+}. 50 ml of IgG (0.5 mg/ml) from patients and controls were added to the cuvettes for 2 minutes incubation, followed by addition of 4-chloro-m-cresol (200 mM) to induce Ca^{2+} release. Curve A shows a normal Ca^{2+} release with IgG from a RyR negative MG patient. Curve B shows that IgG from a Ry1 positive patient strongly inhibits Ca^{2+} release. Curve C shows a normal Ca^{2+} release after removing Ry1 antibodies from the IgG by preincubation with pc2.

Figure 6 shows the 4-chloro-m-cresol induced Ca^{2+} release form SR reacted with IgG from MG patients and controls.

Figure 7 shows the Ca^{2+} release from SR reacted with IgG fractions with and without Ry1 antibodies at different 4-Chloro-m-cresol concentrations. The Ry1 antibodies shift the curve to the right suggesting an allosteric inhibition.

Experimental section

Methods and results

The sera used in the assay

The study included sera from 122 (75) thymoma MG patients (37 Italian, 38 Norwegian, 19 late-onset MG patients and 25 early-onset MG patients (all Norwegian) which had previously been tested in WB for RyR antibodies using a SR preparation as antigen (Mygland et al., 1992; Skeie, G.O., E. Bartoccioni, A. Evoli, J.A. Aarli, and N.E. Gilhus. 1996, *Eur. J. Neurol.* 3: 136-140). The medical records were reviewed and the patients scored according to MG severity at peak of illness and at the last follow-up as previously described (Mygland et al., 1994; Skeie et al., 1996; Skeie, G.O., Å. Mygland, J.A. Aarli, and N.E. Gilhus. 1995, *Autoimmunity* 20: 99-105). In addition sera from 20 Norwegian blood-donors and 3 SLE patients were used as controls.

Biosensor and calcium release

For the biosensor and calcium release assay purified IgG fractions from patient and control sera were obtained using protein G columns according to the protocols provided by Pharmacia AB (Uppsala, Sweden). The IgG fractions were dialysed against HBS buffer and the concentrations adjusted before use to 0.5 mg/ml.

Screening of overlapping RyR fusion proteins

DNA manipulations were carried out as described in Maniatis P, Fritsch EF, Sambrook J. Molecular Cloning. A panel of fusion proteins covering the entire RyR coding sequence were constructed as previously described (Treves S, Chiozzi P, Zorzato F (1993) Biochem J 291, 757-763; Menegazzi P, Larini F, Treves S, Guerrini R, Quadroni M, Zorzato F, (1994) Biochemistry 33, 9078-9084). Gel electrophoresis was carried out as described by Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685. Western blots of bacterial extracts were carried out overnight as described by Gershoni et al., (1985). Indirect

immunoenzymatic staining of Western blots was carried out as described by Young, R.A., B.R. Bloom, C.M. Grosskinsky, J. Ivanyi, D. Thomas, and R.W. Davis. 1985, *Proc. Natl. Acad. Sci. U. S. A.* 82:2583-2587), and detailed by Treves et al., (Treves S, Chiozzi P, Zorzato F (1993) Biochem J 291, 757-763).

RyR fusion protein Western blot

We used the pc2-RyR fusion protein as antigen (Mygland, 1992). Electrophoresis was performed on sodium dodecyl sulphate (SDS) polyacrylamide gels (12%) as described by Laemmli, 1970. 200 ml pc2 (50 mg/ml) were added to 120 ml of sample buffer containing 2% (w/v) SDS, 1.5% (w/v) Tris, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. The mixture was heated to 100°C for 1 minute. About 100 µl of the protein mixture was applied per gel. Proteins separated on the gel were transblotted onto nitrocellulose sheets as described by Towbin, H., T. Staelin, and J. Gordon. 1979, *Proc. Natl. Acad Sci. U.S.A.*76: 4350-4354.

Nitrocellulose sheets were soaked in 5% (w/v) low fat dry milk (Nestle) in phosphate buffered saline (PBS) for 1 hour to block additional protein binding sites. They were washed 3 times in PBS with 0.05% Tween 20 (PBS-Tween), cut into vertical strips and incubated over night at 4 C° with patient and control sera diluted 1:50 in PBS containing 0.5% fat free dry milk and 0.05% Tween 20 (PBS-dry milk Tween (Nestle)). After separate washings for 10 min in PBS-Tween, the nitrocellulose strips were incubated for 1 h with peroxidase-conjugated rabbit antibodies (Dako, Copenhage, Denmark) to human IgG diluted 1:1000 in PBS dry milk Tween. The nitrocellulose strips were then washed and developed in a peroxidase colour development solution containing 30 mg 4-chloro-1-naphthol (Sigma), 17% (v/v) cold methanol, 83% (v/v) PBS and 0.05% (v/v) H_2O_2. Positive

and negative control sera were applied to strips from each transblotted nitrocellulose sheet.

Results

Example 1

The identification of the main immunogenic region of RyR

As shown in figures 1 and 2, MG sera reacted with two of the RyR fusion proteins examined. The reactivity was strongest with the pc2 epitope which is located near the N-terminus. The pc2 epitope comprises residues 799-1152 and consists of 360 amino acids. The pc25 epitope is located between residues 2595-2935. The amino acid sequences (one-letter codes) of the pc2 (SEQ ID NO 1) and pc25 (SEQ ID NO 2) epitopes are given below.

When the pc2 peptide was clipped into 2 fragments (indicated as pc2A and pc2B in figure 3) by Pst I the reactivity with the MG sera was lost indicating that the site for the restriction enzyme is located very near the binding site for the protein, or that this site is important for the conformation recognised by the MG patients antibodies.

Antibodies against pc2 were found in 57 out of 75 thymoma MG patients, 5 out of 19 late-onset MG patients, none of 25 MG hyperplasia patients and none of the 20 blood-donors.

24 of the 44 thymoma MG patients examined for antibodies against pc25 in WB had antibodies reactive with this RyR epitope, but none of 20 MG hyperplasia patients and none of the 20 blood-donors. All patients positive for pc25 had pc2 antibodies while only 24 of the 33 pc2 positive patients had pc25 antibodies.

All sera with reactivity against the full length RyR in Western blots did also react with the pc2 RyR fusion protein (Table 1). The pc2 RyR fusion protein must therefore contain the main immunogenic region.

TABLE 1:

Number of MG patients with Ryl antibodies using different Ryl antigens in WB.

	Thymoma MG	Late-onset MG	Early onset MG
Ryl antibodies (SR)	44/75	0/19	0/25
Ryl antibodies (pc2)	57/75	5/19	0/25
Ryl antibodies (pc25)	24/44	nd	0/25

SR: sarcoplasmic reticulum, pc2: pc2 Ryl fusion protein, pc25: pc25 Ryl fusion protein.

Correlation with titin antibodies

All but 9 thymoma MG patients had titin antibodies. 13 titin positive MG patients had no pc2 RyR antibodies, while 4 patients with pc2 RyR antibodies had no titin antibodies. 5 thymoma MG patients were negative for both titin and pc2 RyR antibodies. 10 out of the 19 late onset sera contained titin antibodies. 5 of the sera also contained MG anti-pc2 antibodies while 5 sera with titin antibodies had no pc2 RyR antibodies.

We established an ELISA (data not shown) using the pc2 fusion protein as antigen. The results must be interpreted together with the Western blot data since some sera negative with the pc2 band in WB had a little background staining of residual bacterial proteins that gave a low positive ELISA signal. The ELISA could therefore be used as a screening test before checking all positive sera for reactivity with the pc2 RyR fusion protein in WB to increase the specificity.

Example 2

Real-time RyR antibody pc2 fusion protein interactions

The reactivity of MG sera with the pc2 fusion protein was studied using a biosensor; BIACORE 1000 (Pharmacia Biosensor AB, Uppsala, Sweden) that allows real time biospecific interaction analysis. This system uses the optical phenomenon of surface plasmon resonance (SPR) that detects changes in optical properties at the surface of a thin gold film on a glass support (sensor ship) (Lofas and Johnsson, 1990). The sensorship is covered by a dextran matrix to which one reactant is covalently linked, while the other(s) is introduced in a flow passing over the surface. The resonance angle depends on the refractive index in the vicinity of the surface, which changes as the concentration of molecules on the surface is modified and is expressed in resonance units (RU). A signal of 1000 RU corresponds approximately to a surface concentration change of 1 ng/mm^2.

Immobilisation of protein to the sensor ship were done via primary amine groups using the amine coupling kit (Pharmacia Biosensor AB) according to standard procedures (Lofas and Johnsson 1990; Fagerstam LG, Frostell A, Karlsson R, Kullman M, Larsson A, Malmquvist M and Butt H. (1990), J Mol Recog. 3, 208-214). The carboxylated matrix of the sensorship CM5 (Pharmacia Biosensor AB) was first activated by injection (Quickinject, flow rate 5 ml/min in HBS pH 7.4 (10 Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% Surfactant P20 (Pharmacia Biosensor AB) of a mixture of NHS/EDS (N-hydroxy-succinimide 50 mM/N-ethyl-N-(3 diethylaminopropyl)-carbodiimide 200 mM) (50 ml). Then in one flowcell 60 ml pc2 RyR fusion protein (1.5 mg/ml in 2.5 mM acetate buffer, pH 4) was shown to give the best binding in preconcentration experiments using buffers of different pH. About 2000 RU were immobilised on the sensorship.

In another flowcell about 4000 RU of an anti-path antibody were immobilised by injecting 70 ml of anti-path antibody (35 ml anti-path 0.5 mg/ml mixed with 35 ml 10 mM acetate buffer, pH 5.5). Residual activated sites were blocked by injection of 50 ml of 1 M ethanolamine hydrochloride pH 8.5 (Pharmacia Biosensor AB).

In one flow cell the sensorship was only activated by NHS/EDS and blocked by ethanolamine hydrochloride without injection of any proteins. This flow cell was used to examine non-specific IgG binding to the dextrane matrix of the sensorship. 50 ml of sera diluted 1:10-1:100 in HBS buffer and purified IgG fractions (dialysed against HBS buffer) (0.5 mg/ml) from MG patients and controls were injected into the flow cells at a constant flow rate of 5 ml/ml and a sensorgram recorded. Between injections the sensor chip was continuously washed with HBS buffer.

Further, different concentrations of anti-path antibody were injected into the flow cell with immobilised pc2 fusion protein to examine the amount and reactivity of the immobilised pc2 protein, and to compare the binding kinetics with the MG sera.

In the flow cell with immobilised anti-path antibodies 50 ml of pc2 fusion protein were injected prior to the injection of sera/IgG fractions. The sensor ship were regenerated by injections of 5 ml of 5% SDS and/or 3 M guanidinium chloride in 5 mM Tris pH 8 between injections of serum or IgG samples.

The curves from sensorgrams obtained by injecting the samples into the flow cell without proteins were subtracted from the curves obtained in flow cells with immobilised pc2 RyR fusion protein to record the specific binding.

Real time Surface Plasmon Resonance Recording

About 2000 RU of the pc2 fusion protein was immobilised on the sensorship. When injecting different concentrations of the high affinity anti-path antibody a

maximal response of 400 RU was obtained. This was therefore the maximal expected response also for the patient sera. The results from injections of 50 ml of purified IgG fractions 0.5 mg/ml from MG patients and controls are shown in Figure 4B. Only IgG fractions from RyR antibody positive MG patients gave a signal above 30 RU (73-360 RU). The sera with the best binding gave a signal close to the maximal expected, indicating that nearly all binding sites for the RyR antibodies were saturated.

The specificity of the interaction was also tested in a sandwich assay. When injecting 50 ml (1 mg/ml) of pc2 RyR fusion protein into the flow cell with immobilised anti-path antibody, 60 RU were bound, as shown in Figure 4C. The second injection of 50 ml RyR antibody positive MG IgG (0.5 mg/ml) gave a signal of about 180 RU (Figure 4C).

The molecular weight of pc2 is 60kD, and the molecular weight of the antibodies is 150 kD. As there is a 1:3 relationship between both the MW and signal in RU there is a 1:1 relationship between the RyR antibodies and the fusion protein i.e. one antibody molecule binds to one RyR fusion protein.

Example 3.

Inhibition of Ca^{2+} release

Ca^{2+} measurements

SR was isolated from white muscles of New Zealand White rabbits and was fractionated into longitudinal tubules and TC in the presence of antiproteolytic agents as described by Saito, A., S. Seiler, A. Chu, and S. Fleischer. 1984, *J. Cell. Biol.* 99:875-885. The SR fractions were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, 100 mM PMSF, 1 mg/ml leupeptin, and were stored in liquid nitrogen until used.

Ca^{2+} release from isolated SR fractions was measured in a Beckmann DU7400 diode array spectrophotometer by

monitoring the A_{710}-A_{790} value of the Ca^{2+} indicator antipyrylazo III (Fluca, Buchs, Switzerland) as described by Palade, P. 1987, *J Biol. Chem.* 262: 6142-6148, and detailed by Treves S, Chiozzi P, Zorzato F (1993), Biochem J 291, 757-763. Pulses of 20 nM Ca^{2+} were administered to load the SR fractions with Ca^{2+}, and the fractions were then incubated with 50 ml of IgG (0.5 mg/ml) from patients and controls for 2 minutes before different concentrations of the Ca^{2+} releasing agent 4-chloro-m-cresol were added. To calibrate the curves 20 nM of Ca^{2+} were added at the end of each experiment.

IgG fractions from patients with RyR antibodies significantly inhibited 4-chloro-m-coractol induced Ca^{2+} release from isolated SR vesicles (Figure 5). The mean Ca^{2+} release rate was significantly lower when the SR vesicles had been incubated with IgG fractions from RyR antibodies positive MG patients (0.93 ± 0.55 mmol Ca^{2+} per mg SR protein per min) compared with IgG fractions from RyR antibody negative MG patients (1.6 ± 0.36 mmol Ca^{2+} per mg SR protein per min) and controls (blood-donors and SLE patients) (1.6 ± 0.21 mmol Ca^{2+} per mg SR protein per min) ($p=0.0021$). The inhibition was concentration dependent (Figure 7) and the curves fitted with a model of allosteric inhibition.

When removing the RyR antibodies by preabsorbing the IgG fractions with the pc2 fusion protein or the SR fractions for 1 hour before using them in the Ca^{2+} release assays the inhibition of Ca^{2+} release disappeared (Figure 5), indicating that the antibodies binding to the pc2 RyR fusion protein are responsible for inhibiting Ca^{2+} release *in vitro*.

Conclusions

By mapping a series of overlapping RyR fusion proteins we have identified the main immunogenic region (MIR) on the RyR for MG patients antibodies. All patients positive for

RyR antibodies in Western blots using a SR preparation as antigen had pc2 antibodies proving that the pc2 fusion protein contains the MIR region. However, 13 patients positive for MIR antibodies were negative in the SR Western blot assay, indicating that the use of the recombinant protein as antigen gives a more sensitive assay. The MIR RyR antibodies were found in 76% of thymoma MG patients but not in blood-donors or young-onset MG patients. The antibodies are therefore closely associated with thymoma MG and the presence of the pc2 fusion protein antibodies strongly suggests the presence of a thymoma. The only patients without a detectable thymoma, positive for the pc2 RyR antibodies, were 5 late-onset MG patients. All of these also had titin antibodies. The late onset MG patients with titin/RyR antibodies are very similar to thymoma MG patients immunologically, clinically and genetically (Aarli, J.A. 1997. Late-onset MG. *Eur. J. Neurol.* 4: 203-209). Microscopic thymomas has been described by Pescarmona, E., S. Rosati, A. Pisacane, E.A. Rendina, F. Venuta, C.D. Baroni. 1992. *Histopathology*. 20: 263-266), and it is not unlikely that the late-onset MG patients with titin and RyR antibodies have a paraneoplastic MG much like the thymoma patients. Some could have a preneoplastic condition; paraneoplastic symptoms often develop years before a tumour is found in a great proportion of patients with other paraneoplastic conditions (Dropcho, E.J. 1998. *Ann. N.Y. Acad. Sci.*: 841:246-261), or the thymoma could have gone into remission in a way similar to that described for other tumours (Dropcho, 1998). RyR antibody positive MG patients should probably be thymectomized regardless of a positive CT scan. The results from the surface plasmon resonance studies showed that there is a strong, specific one-to-one interaction between the MG patients' antibodies and the pc2 RyR fusion protein. The exact binding kinetics of the interaction could not be measured, as the exact concentration of the polyclonal RyR antibodies in the IgG

fractions was unknown. However, using different concentrations of IgG from different patients in curve fitting models were the RyR antibody concentration was set to be 1-10% of the total IgG concentrations, K_D was estimated to be about 10^{-9} (data not shown). Since the Western blot conditions are also for high affinity antibodies, we conclude that the pc2 RyR antibodies are of high affinity.

The pc2 RyR antibodies inhibited cresol activated Ca^{2+} release from isolated SR fractions in a concentration dependant manner suggesting allosteric inhibition. We have previously shown that MG sera containing RyR antibodies inhibit binding of ryanodine to the RyR, which also indicate that the RyR antibodies lock the RyR in the closed position. The RyR antibodies reacting with pc2 were responsible for this effect since the inhibition of Ca^{2+} release disappeared when the pc2 antibodies were removed from the IgG fractions. The pc2 region is located near a clipping site for a protease and therefore probably located on the surface of the cytoplasmic part and the RyR, the foot-region. A portion of this region of the molecule is also interacting with another region on the neighbouring region of the RyR tetramer, and is therefore probably important for the conformation of the receptor (Wu, Y., B. Aghdasi, S.J. Dou, J.Z. Zhang, S.Q. Liu, and S.L. Hamilton. 1997, *J. Biol. Chem.* 272: 25051-25061). A potential calmodulin binding site is also located near the pc2 region. Calmodulin is very important for RyR regulation. The MG patients' RyR antibodies do probably interfere with RyR function; locking the receptor in the closed position; by affecting calmodulin binding or interfering with the "self association between the subunits". By mapping the exact epitope for the MG RyR antibodies one might learn more about RyR function.

Whether antibodies against intracellular molecules are of any pathogenic significance is controversial (Alarcon-

Segovia, D., A. Ruiz-Arguelles, and L. Llorente. 1996, *Immunol. Today*. 17:163-164). Antibodies are able to penetrate the cell membrane and can often be found intracellularly bound to their target antigens (not shown for the RyR antibodies). How they get there and whether they can exert their effector functions is unknown. However, this study shows that the RyR antibodies do affect RyR function directly so if they could also penetrate the cell membrane one would expect a severe effect on muscle function as the antibodies bind to the receptor with high affinity and seem to lock the channel in the closed state thus inhibiting Ca^{2+} release and muscle contraction.

SEQUENCE LISTING

GENERAL INFORMATION:

(110) APPLICANT: UNIFOB, Stiftelsen
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(120) TITLE OF THE INVENTION: Detection of ryanodine receptor antibodies.

(130) FILE REFERENCE:
(A) Medium type: Diskette
(B) Computer: IBM compatible
(C) Operating system: Windows 98
(D) Software: Word 6.0

(150) Earlier patent application:
(A) Application number: NO 19992786
(B) Filing date: 08-JUN-1999

(160) NUMBER OF SEQUENCE ID NOS: 2

(210) INFORMATION FOR SEQ ID NO: 1

(i) Sequence characteristics:

(A) Length: 374 amino acid residues

(B) Type: amino acid

(D) Topology: single

(xi) Sequence description: SEQ ID NO: 1

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            15                  20
Glu Arg Leu Arg Leu Glu Pro Ile Lys Glu
            25                  30
Tyr Arg Arg Glu Gly Pro Arg Gly Pro His
            35                  40
Leu Val Gly Pro Ser Arg Cys Leu Ser His
            45                  50
Thr Asp Phe Val Pro Cys Pro Val Asp Thr
            55                  60
Val Gln Ile Val Leu Pro Pro His Leu Glu
            65                  70
Arg Ile Arg Glu Lys Leu Ala Glu Asn Ile
            75                  80
His Glu Leu Trp Ala Leu Thr Arg Ile Glu
            85                  90
Gln Gly Trp Thr Tyr Gly Pro Val Arg Asp
            95                  100
Asp Asn Lys Arg Leu His Pro Cys Leu Val
            105                 110
Asn Phe His Ser Leu Pro Glu Pro Glu Arg
            115                 120
Asn Tyr Asn Leu Gln Met Ser Gly Glu Thr
            125                 130
Leu Lys Thr Leu Leu Ala Leu Gly Cys His
            135                 140
Val Gly Met Ala Asp Glu Lys Ala Glu Asp
            145                 150
Asn Leu Lys Lys Thr Lys Leu Pro Lys Thr
            155                 160
Tyr Met Met Ser Asn Gly Tyr Lys Pro Ala
            165                 170
Pro Leu Asp Leu Ser His Val Arg Leu Thr
            175                 180
Pro Ala Gln Thr Thr Leu Val Asp Arg Leu
            185                 190
Ala Glu Asn Gly His Asn Val Trp Ala Arg
            195                 200
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Asp Arg Val Ala Gln Gly Trp Ser Tyr Ser
205 210
Ala Val Gln Asp Ile Pro Ala Arg Arg Asn
215 220
Pro Arg Leu Val Pro Tyr Arg Leu Leu Asp
225 230
Glu Ala Thr Lys Arg Ser Asn Arg Asp Ser
235 240
Leu Cys Gln Ala Val Arg Thr Leu Leu Gly
245 250
Tyr Gly Tyr Asn Ile Glu Pro Pro Asp Gln
255 260
Glu Pro Ser Gln Val Glu Asn Gln Ser Arg
265 270
Trp Asp Arg Val Arg Ile Phe Arg Ala Glu
275 280
Lys Ser Tyr Thr Val Gln Ser Gly Arg Trp
285 290
Tyr Phe Glu Phe Glu Ala Val Thr Thr Gly
295 300
Glu Met Arg Val Gly Trp Ala Arg Pro Glu
205 310
Leu Arg Pro Asp Val Glu Leu Gly Ala Asp
315 320
Glu Leu Ala Tyr Val Phe Asn Gly His Arg
325 330
Gly Gln Arg Trp His Leu Gly Ser Glu Pro
335 340
Phe Gly Arg Pro Trp Gln Ser Gly Asp Val
345 350
Val Gly Cys Met Ile Asp Leu Thr Glu Asn
355 360
Thr Ile Ile Phe Thr Leu Asn Gly Glu Val
365 370
Leu Met Ser Asp
374

(210) INFORMATION FOR SEQ ID NO: 2

(i) Sequence characteristics:
(A) Length: 348 amino acid residues
(B) Type: amino acid
(D) Topology: single

(xi) Sequence description: SEQ ID NO: 2

Arg Gly Arg Ser Leu Thr Lys Ala Gln Arg
1 5 10
Asp Val Ile Glu Asp Cys Leu Met Ala Leu
15 20
Cys Arg Tyr Ile Arg Pro Ser Met Leu Gln
25 30
His Leu Leu Arg Arg Leu Val Phe Asp Val
35 40
Pro Ile Leu Asn Glu Phe Ala Lys Met Pro
45 50
Leu Lys Leu Leu Thr Asn His Tyr Glu Arg
55 60
Cys Trp Lys Tyr Tyr Cys Leu Pro Thr Gly
65 70
Trp Ala Asn Phe Gly Val Thr Ser Glu Glu
75 80
Glu Leu His Leu Thr Arg Lys Leu Phe Trp
85 90
Gly Ile Phe Asp Ser Leu Ala His Lys Lys
95 100
Tyr Asp Gln Glu Leu Tyr Arg Met Ala Met
105 110
Pro Cys Leu Cys Ala Ile Ala Gly Ala Leu
115 120
Pro Pro Asp Tyr Val Asp Ala Ser Tyr Ser
125 130
Ser Lys Ala Glu Lys Lys Ala Thr Val Asp
135 140
Ala Glu Gly Asn Phe Asp Pro Arg Pro Val
145 150
Glu Thr Leu Asn Val Ile Ile Pro Glu Lys
155 160
Leu Asp Ser Phe Ile Asn Lys Phe Ala Glu
165 170
Tyr Thr His Glu Lys Trp Ala Phe Asp Lys
175 180
Ile Gln Asn Asn Trp Ser Tyr Gly Glu Asn
185 190
Val Asp Glu Glu Leu Lys Thr His Pro Met
195 200

Leu Arg Pro Tyr Lys Thr Phe Ser Glu Lys
205 210
Asp Lys Glu Ile Tyr Arg Trp Pro Ile Lys
215 220
Glu Ser Leu Lys Ala Met Ile Ala Trp Glu
225 230
Trp Thr Ile Glu Lys Ala Arg Glu Gly Glu
235 240
Glu Glu Arg Thr Glu Lys Lys Lys Thr Arg
245 250
Lys Ile Ser Gln Thr Ala Gln Thr Tyr Asp
255 260
Pro Arg Glu Gly Tyr Asn Pro Gln Pro Pro
265 270
Asp Leu Ser Gly Val Thr Leu Ser Arg Glu
275 280
Leu Gln Ala Met Ala Glu Gln Leu Ala Glu
285 290
Asn Tyr His Asn Thr Trp Gly Arg Lys Lys
295 300
Lys Gln Glu Leu Glu Ala Lys Gly Gly Gly
305 310
Thr His Pro Leu Leu Val Pro Tyr Asp Thr
315 320
Leu Thr Ala Lys Glu Lys Ala Arg Asp Arg
325 330
Glu Lys Ala Gln Glu Leu Leu Lys Phe Leu
335 340
Gln Met Asn Gly Tyr Ala Val Thr
345 348